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RAGE immunoblocking reduces NFkB signaling and increases catalase activity in retina after Endotoxin Induced Uveitis

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Imunobloqueio de RAGE reduz a sinalização de NFkB e aumenta a atividade de catalase na retina após Uveíte induzida por Endotoxina

Trabalho de Conclusão de curso apresentado como requisito parcial para obtenção do título de Bacharel em Ciências Biológicas com ênfase em Biologia Molecular e Celular na Universidade Federal do Rio Grande do Sul.

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Agradecimentos: Primeiramente tenho a agradecer a toda minha família, amigos e amigas que porventura me ajudaram nessa etapa fosse com reflexões importantes para minha formação fosse somente pela presença caridosa nos mais diversos momentos. Mas gostaria de agradecer à algumas pessoas em particular sem especial ordem. Ao professor José Cláudio que durante todos estes anos de laboratório se fez presente não somente como orientador, mas muitas vezes como um exemplo e até mesmo um pai para mim. Ao Uruguaiana que terá sua falta sentida não somente pela personalidade, mas também pela grande pessoa que era. Ao Alexandre que com certeza me mostrou muitos caminhos dentro da biologia, sem contar os inúmeros momentos de auxílio, discussão e confiança. Ao Juciano, que apesar de seu modo xucro e brabo tem um enorme carinho guardado. À Moara que além dos enormes ensinamentos que foste de capaz de me ensinar, nossa personalidade parecida me mostrou muito sobre mim. À minha mãe que sempre compreendeu meus momentos de estresse e muitas vezes relevou minhas grosserias injustificáveis por menor que fossem. Ao meu irmão que apesar de nossas diferenças e desentendimentos sempre saiba que te guardarei no coração com amor e como um exemplo. À Rejane que apesar da minha dificuldade em te mostrar a tua presença e personalidade foram e continuarão sendo de extremo valor para mim.

Dedicatória: Gostaria de dedicar este trabalho a um importantíssimo amigo, talvez não pela colaboração criativa ou desenvolvimento deste trabalho, no entanto a enorme falta que sua presença fará não só para mim, mas com certeza para grandes amigos e pessoas por quem tenho um enorme carinho, será sentida. Não somente teu grande humor cativante e tua maneira extremamente amigável com que tratava certamente a todos, fosse um professor e colega de trabalho, fosse uma pessoa necessitada enquanto tomávamos uma cerveja. Mas vindo de Uruguaiana não podia ser diferente e uma pessoa querida e amável como tu certamente será lembrada, hoje, daqui vinte ou trinta anos; não importa quando for tu sempre vais fazer falta. Fica aqui este trabalho pra ti que em muitos momentos me fez ver que a pesquisa e passar tudo isso adiante não é algo pra gente desistir.
É Rafa, acho que nossos planos vão ter que ficar pra uma outra vinda porquê o potro sem dono despreza a própria morte e leva o destino no peito e contigo a gente pôde perceber que a lua realmente é um tiro ao alvo e quem nasceu pra semente não vai mesmo ficar pra ventania.

Introdução em português: O receptor para compostos finais de glicação avançada *(RAGE)* é um receptor de membrana com múltiplos ligantes dependendo do tecido avaliado. Hoje em dia acredita-se que sua ativação é responsável por sustentar um estado pró-inflamatório no sistema, devido à sua capacidade de ativar cascatas de sinalização como a do NFkB que irão estimular a produção do receptor, produzindo um eixo de *feedback* positivo.

Os sinais intracelulares causados pela administração de Lipopolissacarídeos *(LPS)* são extensivamente usados para simular uma inflamação aguda em mamíferos. Como modelos o LPS já foi descrito para induzir Uveíte Induzida por Endotoxinas *(EIU)*, neurodegeneração, neuroinflamação, sepse e outras patologias. Atualmente, já é sabido que o LPS responde à vias clássicas de sinalização pró-inflamatória, principalmente através de associação com receptores como CD-14 e TLR-4. Aqui buscamos explorar os mecanismos de inflamação causada por LPS mediadas pela via do RAGE e suas possíveis consequências sobre a sinalização pró-inflamatória e o estresse oxidativo num modelo animal de EIU.

Originalmente o RAGE foi descrito como um receptor para compostos avançados de glicação *(AGES)*, porém estudos das últimas décadas sustentam a ideia de que o RAGE possui uma maior diversidade de ligantes capazes de ativar os seus mecanismos de sinalização intracelular que à longo prazo são capazes de aumentar o próprio conteúdo de RAGE na membrana, assim gerando o seu eixo de retroalimentação positiva. Sabendo o papel do RAGE em sustentar a lesão inflamatória e sua própria expressão, torna-se importante o desenvolvimento de métodos terapêuticos que abordem as vias não clássicas de sinalização inflamatória, como a do RAGE.

Poucos trabalhos reportaram o papel do RAGE em um contexto de Uveíte e a grande maioria dos trabalhos buscou localizar e estudar o receptor num contexto diabético uma vez que este foi primeiramente descrito por sua capacidade de se ligar à AGES e que estes são responsáveis em boa parte pela fisiopatologia da diabetes.

Alguns autores já relataram a importância do RAGE e seus ligantes em doenças retinais, devido ao papel da sinalização pró-inflamatória envolvida em diversas doenças. A uveíte é uma doença intraocular inflamatória que pode levar a sérias complicações, sendo a responsável por cerca de 15% dos casos de cegueira e 20% dos casos de cegueira-legal ao redor do mundo. Corticosteróides são as principais estratégias utilizadas para o tratamento da doença. No entanto, já se sabe que seu uso pode causar diversos efeitos sistêmicos indesejáveis e também efeitos oculares indesejáveis, como a formação acelerada de catarata e aumento da pressão intraocular.

O processo de antagonismo do RAGE através de anticorpos ou do sequestro de seus ligantes já se demonstrou efetivo em proteger os insultos causados pelo LPS e pela sepse em modelos animais. Até onde sabemos, atualmente, somente um trabalho publicado por Watanabe em 2009 explorou o receptor em um modelo de uveíte, porém este utilizou antígenos oculares para causar uma Uveíte Autoimune *(EAU)*. Outros trabalhos incluindo alguns publicados pelo nosso grupo, já foram capazes de demonstrar a capacidade do antagonismo de RAGE em suprimir as respostas pró-inflamatórias e os marcadores de dano oxidativo em modelos de inflamação sistêmica causada por LPS.

Devido a capacidade do RAGE em sustentar a lesão inflamatória e a falta de estudos explorando este receptor em condições de inflamação intraocular, nós buscamos avaliar os efeitos do antagonismo de RAGE prévio à um insulto causado pela administração sistêmica de LPS, simulando um estado de EIU no modelo animal aqui proposto. Buscamos explorar vias clássica de sinalização pró-inflamatória assim como parâmetros de estresse oxidativo e a presença de seus produtos finais diretamente no tecido extraído da retina.

Aqui exploramos a atividade de Catalase *(CAT)*, responsável pela detoxificação de peróxido de hidrogênio, possíveis alterações na atividade desta enzima poderiam nos indicar um desequilíbrio redox no sistema causado tanto pela inflamação gerada quanto pela presença de LPS circulante. Buscamos também explorar o conteúdo proteica de certos produtos finais do estresse oxidativo, como nitrotirosina e 4-hidróxinonenal. De forma a avaliar um possível acúmulo destes produtos diretamente no tecido da retina.

Como forma de avaliar a lesão inflamatória, também avaliamos a fosforilação de certas proteínas relacionadas à ativação de RAGE e sinalização de NFkB por Western Blotting. Escolhemos analisar as isoformas fosforiladas e totais de: ERK 1/2; Stat3 e p65. Assim como o conteúdo de RAGE após o insulto causado por LPS, de maneira que poderíamos supor um maior conteúdo de RAGE causado pela elevada transcrição de NFkB, que por sua vez estimula a produção de RAGE para a membrana.

Além disso, como não encontramos nenhuma ilustração publicada que buscasse elucidar a sinalização pró-inflamatória num contexto de uveíte mediada por RAGE, tomamos a liberdade de criar um diagrama que demonstrasse os processos de sinalização vistos neste trabalho através do software Adobe Illustrator. Novamente é importante ressaltar que o RAGE não é o único ligante extracelular de LPS e que certamente outros receptores como CD-14 e TLR-4 também podem ser responsáveis, pelo menos em parte, pela sinalização pró-

inflamatória vista aqui. Porém, a capacidade do RAGE em sustentar a lesão inflamatória devido ao potencial do RAGE em elevar seu próprio conteúdo pelo estímulo ao NFkB.

RAGE immunoblocking reduces NFkB signaling and increases catalase activity in retina after Endotoxin Induced Uveitis

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Abstract:

Aim of the study: The receptor for advanced glycation end products *(RAGE)* is a multiligand membrane receptor associated with a variety of roles depending on the tissue evaluated and its activation is believed to sustain a proinflammatory state in the system. Also, it has been shown that blockage of RAGE can somehow display a protective effect against insults induced by Lipopolysaccharide *(LPS)* endotoxemia. LPS is widely used to mimic strong and acute inflammation in mammals and also as a model for Endotoxin Induced Uveitis *(EIU)* in rodents. **Materials and Methods:** In this work we evaluated if pre-treatment with *anti-RAGE IgG* could have a protective effect in the retinas of Wistar Rats. Animals (60 days old) were randomly distributed into 2 treated groups and 2 control groups and had their retinas collected 24 hours after induction of EIU via LPS endotoxemia. *Catalase activity* and also the levels of protein and lipid oxidation were used as oxidative stress markers and some RAGE downstream signaling molecules were quantified by western blotting. **Results:** We observed that RAGE blockage associated with LPS reduced the phosphorylation of *ERK1/2*, *Stat3* and *P65* and increased Catalase activity while not altering oxidative damage markers. **Conclusion:** Our results indicate us that RAGE has a pivotal role in retinal tissue mainly mediating signal transducing factors. Oxidative damage markers were not elevated in retinal tissue however this may be a time-dependent issue since we used a short-term protocol for evaluating LPS endotoxemia and phosphorylation of NFKB ligands was observed.

Keywords: Endotoxin Induced Uveitis, anti-RAGE IgG, retinal tissue, Catalase activity, NFKB signaling,

1.Introduction:

The receptor for advanced glycation end products (RAGE) is a multiligand membrane receptor that exerts crucial roles in the development of proinflammatory processes [1]. RAGE is associated with distinct cell responses depending on the tissue of origin and developmental stage [2], also RAGE is classified as a damage-associated molecular pattern (DAMP) receptor because molecules with pro-inflammatory or pro-apoptotic activities were observed as its ligands. The activation of RAGE induces the expression of pro-inflammatory cytokines and also the activation of NADPH oxidase, stimulating production of reactive species, which may increase the oxidative damage to biomolecules sustaining local inflammation and tissue damage [3]. When up-regulated by DAMPs RAGE-activated signaling induces the transcription of proinflammatory cytokines and enhances its own transcription, establishing a positive feedback axis of proinflammatory signaling [4]. As stated by [2], many authors have documented the role of RAGE in sustaining the proinflammatory state in some chronic diseases due to the capacity of RAGE to enhance its own expression when activated. Also, in a methodological article, published by Umesh & Kota in 2019 the authors tells us there is a growing body of evidence supporting the association between oxidative stress and inflammation in the uveitis pathophysiology [5].

Not many studies have reported the role of RAGE in either EIU or Experimental Autoimmune Uveitis (EAU). Watanabe (2009) and colaborators have been able to locate through immunofluorescence staining the presence of RAGE on both posterior and anterior cells extracted from Lewis Rats in EAU model. RAGE positive macrophages were significantly increased in EAU animals when compared to non-treated rats, indicating that RAGE is highly expressed in active EAU [6].

Barile & Schmidt wrote on the impact of studies of the RAGE-axis ligands and the signaling pathways and perturbations in several cells of the diabetic retina, not only due to the hyperglycemic condition present in the diabetes pathology but also due to the importance of inflammatory mechanisms in several retinal diseases [7].

Nowadays it is known that RAGE has many ligands depending on tissue and cell evaluated, as researches have linked retinal complications to the proinflammatory signals involved it is important to notice that RAGE surely has a role in many retinal diseases [7]. In the eye, RAGE has been identified in several neural cells but mainly in vascular and retinal pigment epithelium cells located in the posterior portion of the eye-globe, surrounding the systemic vasculature, choroid and the Blood-Ocular-Barrier.

Uveitis is an intraocular inflammatory disease that can lead to serious and several complications. According to [8] and the WHO, uveitis accounts for 10-15% of the cases of total blindness and up to 20% of legal blindness cases, being the fifth most common cause of visual loss in the developed world and leading to devastating visual loss.

The RAGE blocking processes via antibodies or kidnapping its ligands via its soluble isoform has shown to be protective against systemic insults induced by sepsis and LPS endotoxemia [9, 10, 11]. Lipopolysaccharides, or LPS, are a known group of endotoxins found on the outer membrane of gram—negative bacteria and it is extensively used to mimic strong and acute inflammatory responses in mammals; leading to a range of behavioral and endocrine alterations regulated in the Central Nervous System collectively known as host response inflammation [12]. Its effects are regulated by proinflammatory cytokines like Interleucin-1β and Tumor Necrosis Factor-α released in response to LPS endotoxemia [13]. LPS activates a range of kinases and NFKB, molecules related to the release of cytotoxic factors such as oxidative stress markers and cytokines.

The cellular infiltration recruited by the uveitis pathophysiology ultimately shall cause the breakdown of the blood-aqueous barrier leading to increased protein permeability and elevated presence of proinflammatory cytokines like TnF- α and IL-6, and cell adhesion molecules like those from the family of Selectins.

The presence of endotoxins, like LPS, into the blood characterizes the endotoxemia and leads to reaction of the immune system classically mediated by the complex of CD-14 and Toll-Like Receptor-4 responsible for activating and recruiting immune cells like macrophages and extremely lead to septic-shock if the endogenous immune response is impaired [14]. However, in the last decades a lot of works have classified RAGE as a receptor capable of binding and signaling by LPS.

The blood-aqueous and blood-retinal epitheliums are part of the blood-ocular barrier, which is the main physiological barrier between the local blood vessels and most parts of the eye, its role is to stop and prevent many toxic compounds like LPS and drugs from passing through it. One of its roles is to stop these toxic molecules from reaching the functional ocular structures where they can cause visual impairment and vision loss [15]. Corticosteroids are the main therapeutic strategy to combat uveitis, suppressing inflammation and modulating the host's stress and immune responses. Corticosteroids use can lead to many unwanted ocular side effects like accelerated cataract formation and increased intraocular pressure while also presenting systemic unwanted side effects like hypertension, diabetes, Cushing's syndrome

and osteoporosis [16]. These factors make the development of preventive agents for uveitis and the mechanisms of intraocular inflammation matters to be explored.

The aim of our work was to investigate anti-RAGE IgG administration in a short-term systemic inflammation induced by LPS administration, simulating a state of endotoxin induced uveitis (EIU). According to [5] animal models to study uveitis pathophysiology were not available until the 80s when other authors described that systemic immunization with endotoxins lead to bilateral acute anterior uveitis in rodents. EIU is an animal model to study acute form of uveitis that can be induced in rodents using a sub-lethal dose of exogenous bacterial toxins such as LPS.

Systemic LPS is known to induce peripheral inflammation, oxidative stress and neuroinflammation due to leakage of the Blood-Brain-Barrier [17, 18], furthermore, LPS is known to activate macrophages leading to the production of pro-inflammatory cytokines like IL-1β and TnF-α, increasing the generation of reactive species [19], which leads to the accumulation of physiological markers of theirs processing such as nitrotyrosine and lipid peroxidation products and an increase in Catalase activity.

Also in Gasparin's review about the experimental animal models to study uveitis pathophysiology authors wrote that EIU can be induced with low doses of endotoxins, such as LPS, by intravenous, intraperitoneally or subcutaneous administration. In rats the ocular inflammatory signs appear few hour after LPS injection and may spontaneously resolve within days [20]. With these evidence and since the LPS models are known to have a high mortality rate, we chose to evaluate retina's 24-hours after the first LPS injection.

2.Material and Methodology:

2.1.Reagents and antibodies:

Glycine, bile salts and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich® (St. Louis, USA). Electrophoresis and immunoblot apparatus and reagents were bought from Bio-Rad (Hercules, USA) and GE Healthcare Brazilian Headquarter (Sao Paulo, Brazil). Polyclonal and monoclonal antibodies from Cell Signaling Technology® (MA, USA) were: p-ERK-44/42 (Thr202/Tyr204), ERK-44/42, NFκB-p65, p-NFκB-p65, STAT3 and p-STAT3. Anti-β-actin was from Sigma-Aldrich® (St. Louis, USA). Antibodies against nitrotyrosine and 4-hydroxynonenal were obtained from Abcam® (Cambridge, UK). Anti-RAGE rat IgG from Santa Cruz Biotechnology, Inc. (Texas, USA) was used for RAGE blocking and other

techniques. Anti-rabbit IgG, peroxidase conjugated and anti-mouse IgG, peroxidase conjugated were from Merck Millipore (MA, EUA). Antibodies dilution are described in the techniques and several information regarding their isotype, catalog number as well as Host Species and Reactivity are described in the Supplementary Table 1. Immunoblot chemiluminescence detection was carried out with the West Pico detection kit from Thermo Scientific Pierce Protein Biology Products (Illinois, USA).

2.2.Animals and treatment:

Male Wistar rats (60-days old) were obtained from our breeding colony (*n*=6 per group). They were caged in groups of four animals with free access (*ad libitum)* to water and standard commercial food (Chow Nuvilab CR-1 type; Curitiba, PR, Brazil). Rats were maintained in a twelve-hour light-dark cycle in a temperature-controlled colony room (24±2°C). Animals were handled for 7 days before the procedures to reduce daily manipulation and weighting stress.

EIU was induced using a single dose of LPS administered intraperitoneally. Rats were separated into four groups: *a)* control group – received one saline injection and one hour later a second saline injection; *b)* RAGE group – received one RAGE antibody (RAGE-Ab) injection and one hour later a saline injection; *c)* LPS group – received one saline injection and one hour later one LPS injection; *d)* RAGE+LPS group - received one RAGE-Ab injection and one hour later one LPS injection. All injections were intraperitoneally administered. RAGE antibody groups received a 50 μg/kg RAGE-Ab dose based on previous reports [2, 19]. For LPS groups a dose of 5mg/kg was used, which is considered a high dose of LPS and was used on our previous study [2].

As a control to investigate possible interferences of RAGE IgG we used an anti- rat IgG conjugated with Alexa Fluor 555 (Thermo Fisher, A20187) in a dose of 50 μg/kg before LPS injection to evaluate ERK/p-ERK signaling. It's worth to notice that since no alterations have been found these data was omitted. This methodology has been well described by [2] in our previous studies using RAGE-Ab and LPS models.

After 24 hours, animals were anesthetized and the retinas from each eye were dissected from the eye-globe under a stereomicroscope into a saline rinsed Petri dish and stored at -80ºC for posterior analysis. One retina from each rat was homogenized in Phosphate Buffer Saline and used in Catalase and indirect-ELISA, the other retinas were used for Western Blotting experiments.

All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health (NIH, 1985) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. Our research protocol was approved by the Ethical Committee for Animal Experimentation of the Universidade Federal do Rio Grande do Sul- Brazil (CEUA-UFRGS) under the project number #27683.

2.3.Protein Extraction and Quantification:

Protein was extracted using 400µl Phosphate Buffer Saline *(PBS)* or a radioimmunoprecipitation assay buffer and a glass mortar and pestle, samples were homogenized by hand to avoid excessive air bubbles formation and then transferred to a new sterile microtube where they were centrifuged at 10.000g for 10 minutes and supernatant was transferred to a new tube prior quantification. Total protein content of the homogenates were quantified by Bradford method and used for data normalization [21].

2.4.Enzyme-linked immunosorbent assay (ELISA):

Specific ELISA plates purchased from *Greiner Bio-One* were used. TMB substrate solution was purchased from *Thermo Fisher*, catalog number #002023. Tween-20 for preparation of Wash Buffer was acquired from *Kasvi*, catalog number #K9-9191. All antibodies used are described in Supplementary Table 1 containing all information regarding host species and reactivity..

Samples were homogenized and normalized in phosphate buffer 50 mM (KH_2PO_4 and K_2HPO_4, pH 7.4). The final concentration used for all the samples was 10µg/ml The homogenate samples were placed in ELISA plates and incubated 24 hours and then washed three times with Wash Buffer (PBS 10mM, 150mM NaCl and 0,05% Tween-20). Subsequently, 200 µL of primary antibody (1:5000) was added and incubation was carried for 2 hours at room temperature. The plates were washed three times with Wash Buffer and incubated with the specific (according to fabricant) IgG peroxidase-linked secondary antibody (1:2000) for 2 hours. After washing the plate three times with Wash Buffer, 100 µL of substrate solution (TMB spectrophotometric ELISA detection kit) were added to each well and incubated for 10 min. The reaction was terminated with 50 µL/well of 2 M sulfuric acid stopping reagent and the absorbance read at 450 nm in a spectrophotometer (SpectraMax® i3). The results are expressed in relative percentage to control groups [2].

2.5.Western Blotting:

To perform immunoblot experiments, the tissues were prepared and normalized using an radioimmunoprecipitation assay buffer protocol [22]. The proteins (30 μg/well) were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes with Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, CA, USA). Protein loading and electroblotting efficiency were verified through Ponceau S staining, and the membranes were washed with TTBS. Membranes were incubated for 20 min at room temperature in SNAP i.d. 2.0 Protein Detection System (Merck Millipore, MA, USA) with each primary antibody (1:500 dilution) and subsequently washed with TTBS. Anti-rabbit or mouse peroxidase-linked secondary antibody was incubated for an additional 20 min in SNAP (1:5.000 dilution) and washed again. The immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit. The chemiluminescence was captured with an ImageQuant LAS 4000 (GE Healthcare, São Paulo, Brazil). Densitometric analysis of the images was performed using ImageJ software (ImageJ v1.49, National Institute of Health, USA). Blots were developed to be linear in the range used for densitometry. All results were expressed as a relative ratio to β-actin or total isoform [2].

2.6.Catalase activity:

Catalase activity was evaluated by the ratio of H_2O_2 absorbance decrease as it converts peroxide to oxygen and hydrogen in optimum temperature, when one unit of catalase degrades 1 μmol H_2O_2 per minute [23]. Briefly PBS prepared samples were normalized to 1μg/μl and 10μg were transferred to a 96-well Greiner Plate, in which 185μl of PBS and 5μl of H_2O_2 1M was added and then read at 240nm and temperature of 37ºC during 5 minutes using kinetics.

2.7.Statistics:

Statistical analysis was performed with GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego, USA). Data were evaluated by one-way ANOVA analysis and followed by Tukey's Multiple Comparison *post-hoc* test. Differences were considered significant when $p<0.05$.

3.Results:

In Figure 1 our results show an increase of Catalase activity *(A)* on RAGE+LPS group not seen in other animal's retinas which presented a basal Catalase activity around 1CAT Unit per milligram of protein.

Evaluating oxidative stress markers for protein damage via nytrotyrosine *(B)* we found no significant nytrotyrosine levels between the treatments, however LPS treatment presented the highest variation amongst groups. To access lipoperoxidation related oxidative stress we used the marker 4-hydroxynonenal *(C),* here we show that RAGE pre-treatment reduced lipoperoxidation in retinal region tissue when compared to both Control and LPS groups. RAGE+LPS treatment did not reduced 4-hydroxynonenal levels as expected.

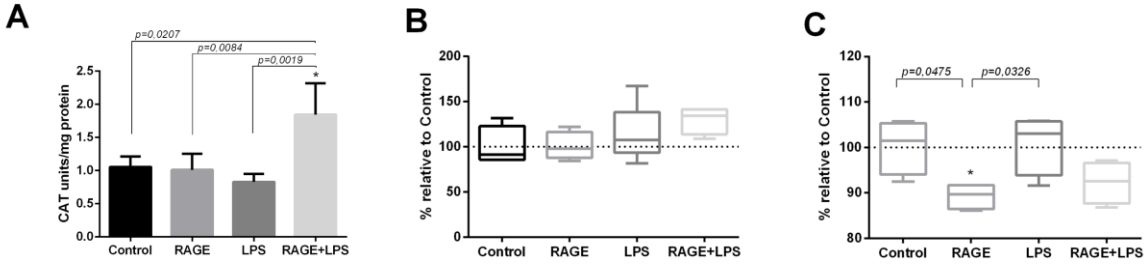

Figure 1 Oxidative stress evaluation: Through spectrophotometry we analyzed Catalase Activity (A) and oxidative stress products Nytrotyrosine (B) and 4-hydroxynonenal (C) via indirect-ELISA. Ours results indicate higher CAT in RAGE+LPS treatment and a lower 4-HNE in RAGE group. RAGE immunoblocking was not able to reduce oxidative damage markers after EIU. Sample Size = 6 animals per group.

Western blotting was used to evaluate the immunocontent of some proteins related to LPS endotoxemia (Figure 2). LPS treated group presented higher phosphorylation of ERK ½ (A), Stat3 (B) and p65 (C). The treatment with RAGE was able to inhibit these protein phosphorylation and possibly blocked the signaling pathways to NFKB transcription

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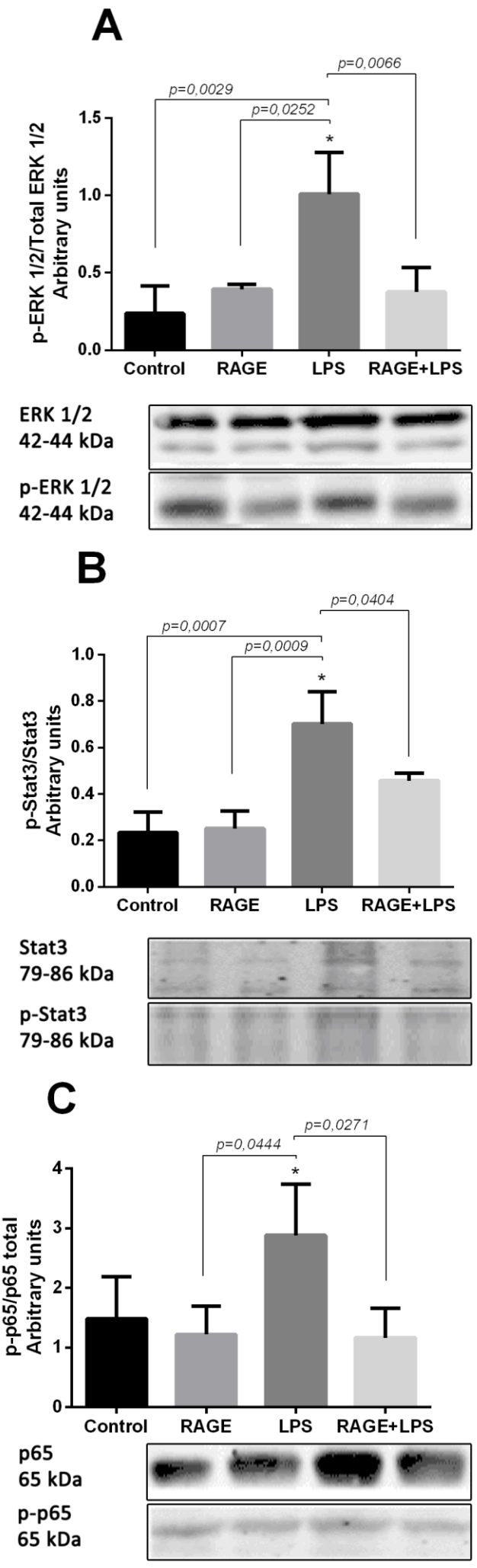
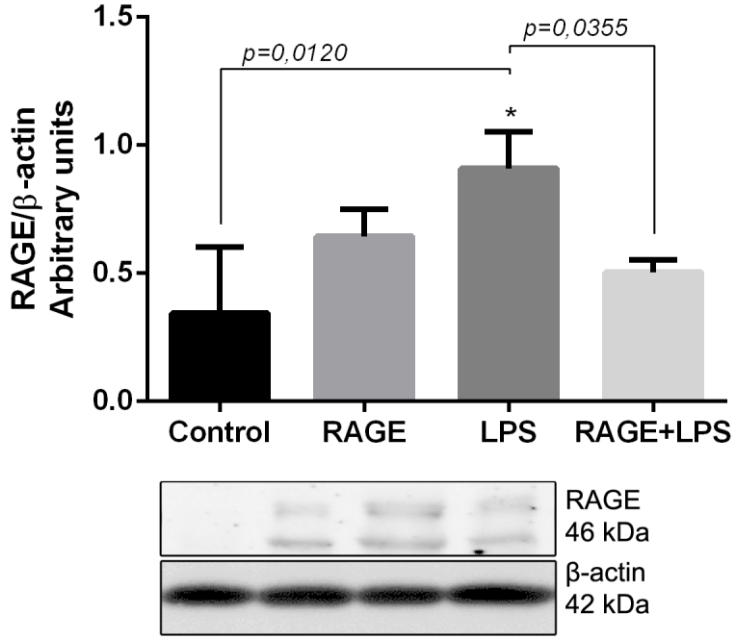
Figure 2 Western Blot experiments related to proinflammatory and NFkB signals: Investigation of total and phosphorylated proteins in NFkB related signaling proteins demonstrated elevated phosphorylation of ERK1/2; Stat3 and p65 in LPS group. These results indicate lower expression of RAGE mainly by signals that mediate NFkB signaling. Sample Size = 3 (Control and RAGE groups); Sample Size = 4 (LPS and RAGE+LPS groups). Arbitrary Units represent phosphorylated units divided by their total isoform.



RAGE content was also evaluated to check for increased membrane RAGE due to NFkB phosphorylation, leading to RAGE production and incrementing its positive feedback axis. As expected RAGE content was increased in LPS group when compared to both Control and RAGE+LPS groups, however this difference was not statistical significant in RAGE group when compared to LPS animals.



Supplementary Figure 1 Western Blot experiment to analyze RAGE content: RAGE immunocontent was found elevated in LPS group when compared to both Control and RAGE+LPS groups. This indicates RAGE expression is increased in an EIU condition and that RAGE immunoblocking is able to mediate its positive feedback axis possibly due to NFkB transcription inducing RAGE production and recruitment to the membrane. Sample Size = 3 (Control and RAGE groups); Sample Size = 4 (LPS and RAGE+LPS groups). Arbitrary units represent RAGE immunocontent divided by β-actin immunocontent; β-actin was also added as a demonstration of pipetting and protein dosage accuracy.

4.Discussion:

Systemic inflammation induced with LPS is widely used as standard protocol to induce neuroinflammation and neurodegeneration [18]. LPS is also an inductor of infection/inflammation widely used in animal models to study proinflammatory mechanisms in different cells from different tissues [5, 12, 17, 18, 19]. Previous results published by our group [2, 24] show us that LPS induces an enhanced inflammatory response in rats and also induces RAGE activation, in turn improving the inflammatory responses induced by LPS.

LPS has other known ligands that shall lead to inflammation mediated via NFkB, the most extensively studied complex for LPS binding is CD-14 and Toll-like receptor-4 mediated signaling. NFkB phosphorylation mediated by RAGE is a somewhat new topic and for years the receptor has been studied in close relation to the diabetes pathology and Advanced-Glycation End-products accumulation, hence its name. RAGE's positive-feedback axis of signaling demonstrates the importance of therapeutic agents able to slow down its continuous inflammatory state or at least inhibit some of the signaling responses of this LPS receptor. The classical RAGE's response to LPS signaling can be ultimately seen as oxidative stress markers and elevated proinflammatory cytokynes or earlier as elevated signaling of damage related pathways like phosphorylation of NFkB related proteins that shall lead to an intense and continuous inflammatory state if mediated via RAGE, due to its capability of producing a positive feedback axis.

However, the way these proinflammatory mechanisms signaling occurs must be clarified so efficient therapeutic methods can be employed. RAGE blockage inhibits intracellular proinflammatory signaling cascades, which on the long term are able to induce cell death through oxidative damage and then systemically target other tissues [2, 3, 9].

In this study we used anti-RAGE IgG treatment before the LPS stimuli to investigate the effects of immunoblocking RAGE in proinflammatory pathways and intracellular signaling processes induced by EIU in retinal tissue.

Catalase is used to decrease hydrogen peroxide levels inside the cell. Elevated CAT activity on RAGE+LPS group can indicate us a higher response to injury into retinas due to elevated CAT activity, on the other hand, the lower CAT activity found on LPS group can indicate that these animals are having impairment into their endogenous detoxifying mechanisms. Defects on detoxifying mechanisms can lead to the production of ROS and in due time shall lead to oxidative stress.

Our previous results from Gasparotto et al. showed that LPS (5 mg/Kg) led to increased serum levels of both 4-hydroxynonenal and nytrotyrosine consistently with an oxidative stress response. Pre-treatment with anti-RAGE IgG wasn't able to decrease these markers in our RAGE+LPS group, however this may be a time dependent issue, since pro-inflammatory signaling was enhanced and CAT activity was impaired. In the present study we did not found the same consistent response analyzed on serum by Gasparotto et al. in the retinal homogenate.

Stat3 phosphorylation is found following central nervous system injury. The increase in reactive gliosis via JAK/Stat3 after intraperitoneal injection of LPS elevating serum and retinal levels of pro-inflammatory cytokines is documented by [12, 25]. Knowing the role of Stat3 in production of pro-inflammatory cytokines and ROS, which in turn can increase the oxidative stress response and the inflammatory lesion [26, 27] we decided to access Stat3 phosphorylation and a possible role of RAGE in regulating this pathway. Hydrogen peroxide can modulate Stat3 phosphorylation when catalase activity is inhibited [26]. Our results showed similar results found by Peña et al.

RAGE+LPS treatment increased CAT activity, suggesting an increase of redox defenses. High levels in CAT activity are important to decrease hydrogen peroxide levels, which in turn reduce the oxidative stress and inhibits previously described signaling cascades like that from Stat3.

P65 is an NFkB subunit which is indispensable for several cellular processes under physiologic conditions. Overexpression of this complex can lead to an extensive pro-inflammatory state with varying molecular mechanisms between cells [28], we found that phosphorylated p65 levels were lower in RAGE +LPS animals compared to LPS animals indicating that RAGE immunoblocking is able to regulate the NFkB signaling via the receptor in retinal tissue.

In Figure 3 we have created a diagram illustrating RAGE mediated signaling via the proteins analyzed. The EIU model here proposed via LPS injection presented three proinflammatory intracellular targets with elevated phosphorylation, all related to NFkB phosphorylation and expression and to RAGE's continuous proinflammatory feedback axis. Since other receptors and not only RAGE is able to activate NFkB transcription due to circulating LPS reaction, several intracellular processes are not here described and signaling pathways omitted since they were not the focus of the study. Nonetheless, we tried to illustrate some of RAGE's signaling mechanisms in EIU condition, while also trying to elucidate a possible role in RAGE antagonism as a therapeutic target for future studies.

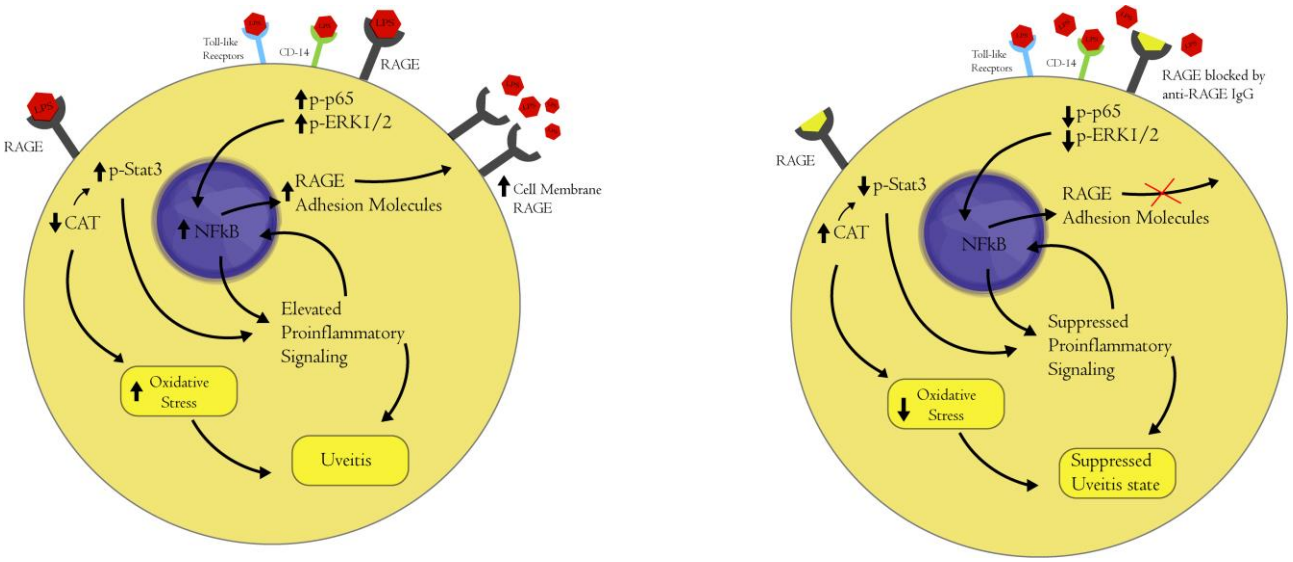


Figure 3 Diagram of RAGE signaling during EIU and after RAGE immunoblocking in EIU condition: Although other ligands are able to bind to LPS and lead to NFkB transcription, RAGE is the only know LPS ligand able to produce a positive feedback axis that shall lead to a continuous inflammatory state. In this study we used anti-RAGE IgG for RAGE immunoblocking and have found that proinflammatory signaling was suppressed and Catalase Activity was improved, leading to a suppressed Uveitis state after RAGE antagonism in an EIU experimental model.

Chen et al. suggests that suppression of NFkB can be a potential therapeutic target for ocular inflammation. NFkB activation also plays a key role in LPS-dependent gene expression, this activation will induce production and release of both inflammatory mediators and adhesion molecules [16].

5.Conclusion:

Here we used an acute inflammation protocol with a single dose of LPS to simulate endotoxin induced uveitis and evaluate short-term effects directly on retinal tissue. Based on our results, RAGE has critical role in retinal signaling during EIU, mainly by mediating proinflammatory signaling cascade resulting in neuronal impairment and continuous inflammatory state. Oxidative damage markers had no alteration in retina, however it is probably a time-dependent issue, since there is phosphorylation of NFkB pathway proteins, therefore other times will be investigated in future experiments.

RAGE activation and signaling via LPS will lead to oxidative stress and a continuous inflammatory state, but RAGE is not the only membrane receptor that LPS can bind and so at

least some level of NFkB phosphorylation is occurring due to CD-14 and other Toll-like Receptors that lead to phosphorylation of several different proteins not observed here like IKB, PI3K and some MAP-kinases..

We did observe that RAGE's activation will increase phosphorylation of at least three related molecules: p65; ERK 1/2; and Stat3. ERK 1/2 will regulate cytokine production and release, incrementing the inflammatory state. Phosphorylated p65 will induce NFkB transcription inside the nucleus, which promotes increased levels of proinflammatory cytokines and adhesion molecules and that of RAGE itself in the cellular membrane. Both increased levels of RAGE and adhesion molecules will also promote the extent of the inflammatory lesion. Stat3 phosphorylation promotes an increase in proinflammatory cytokine levels, affecting both NFkB transcription and RAGE expression, also due to its positive feedback axis.

Low CAT activity indicates elevated Hydrogen Peroxide levels, which will increase Stat3 phosphorylation in turn augmenting proinflammatory cytokine levels like Peña and colleagues described. Both low CAT activity and elevated proinflammatory cytokines expression shall lead to a state of oxidative stress inside the cell. Together with the extensive and continuous inflammatory state due to RAGE's positive feedback axis, both oxidative stress and elevated proinflammatory signaling have pivotal roles in the Uveitis pathophysiology mainly via signaling pathways in which NFkB transcription exerts the lead role.

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7.Conflicts of Interest:

The authors declare no conflicts of interest.

8. Informed Consent:

Informed consent was obtained from all individual participants included in the study.

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Figure 1 Oxidative stress evaluation: Through spectrophotometry we analyzed Catalase Activity (A) and oxidative stress products Nytrotyrosine (B) and 4-hydroxynonenal (C) via indirect-ELISA. Ours results indicate higher CAT in RAGE+LPS treatment and a lower 4-HNE in RAGE group. RAGE immunoblocking was not able to reduce oxidative damage markers after EIU. Sample Size = 6 animals per group.

Figure 2 Western Blot experiments related to proinflammatory and NFkB signals: Investigation of total and phosphorylated proteins in NFkB related signaling proteins demonstrated elevated phosphorylation of ERK1/2; Stat3 and p65 in LPS group. These results indicate lower expression of RAGE mainly by signals that mediate NFkB signaling. Sample Size = 3 (Control and RAGE groups); Sample Size = 4 (LPS and RAGE+LPS groups). Arbitrary Units represent phosphorylated units divided by their total isoform.

Figure 3 Diagram of RAGE signaling during EIU and after RAGE immunoblocking in EIU condition: Although other ligands are able to bind to LPS and lead to NFkB transcription, RAGE is the only know LPS ligand able to produce a positive feedback axis that shall lead to a continuous inflammatory state. In this study we used anti-RAGE IgG for RAGE immunoblocking and have found that proinflammatory signaling was suppressed and Catalase Activity was improved, leading to a suppressed Uveitis state after RAGE antagonism in an EIU experimental model.

Supplementary Figure 1 Western Blot experiment to analyze RAGE content: RAGE immunocontent was found elevated in LPS group when compared to both Control and RAGE+LPS groups. This indicates RAGE expression is increased in an EIU condition and that RAGE immunoblocking is able to mediate its positive feedback axis possibly due to NFkB transcription inducing RAGE production and recruitment to the membrane. Sample Size = 3 (Control and RAGE groups); Sample Size = 4 (LPS and RAGE+LPS groups). Arbitrary units represent RAGE immunocontent divided by β-actin immunocontent; β-actin was also added as a demonstration of pipetting and protein dosage accuracy.

Antibody	Reactivity	Host Species	Isotype	Catalog number
4-HNE	Independent	Rabbit	IgG	#Ab46545
β-actin	Mouse, Rat, Human, +	Mouse	IgG	#A1978
ERK 44/42	Mouse, Rat, Human, +	Rabbit	IgG	#CS9102
NOS	Independent	Mouse	IgG	#Ab7048
p65	Mouse, Rat, Human, +	Mouse	IgG	#CS6965
p-ERK 44/42	Mouse, Rat, Human, +	Rabbit	IgG	#CS9101
p-p65	Mouse, Rat, Human, +	Rabbit	IgG	#CS3033S
p-Stat3	Mouse, Rat, Human, +	Rabbit	IgG	#CS9131S
RAGE	Mouse, Rat, Human	Rabbit	IgG	#SC5563
Stat3	Mouse, Rat, Human, +	Mouse	IgG	#CS9139S
Rabbit Secondary	Rabbit	Goat	IgG	#AP132P
Mouse Secondary	Mouse	Goat	IgG	#AP124P

Supplementary Table 1 This table contains all relevant information about the antibodies used in the study. Name, Reactivity, Host Species, Isotype and Catalog Number. Dilution used for each antibody are described in the methodology used for the experiment.